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PROVISIONAL SPECIFICATION

Invention Title: "A TREATMENT"

The invention is described in the following statement:

TITLE

"A TREATMENT"

FIELD OF THE INVENTION

THIS INVENTION relates to a method of treating sufferers of Multiple Sclerosis. This invention also relates to a pharmaceutical composition for treating Multiple Sclerosis.

BACKGROUND OF THE INVENTION

Multiple Sclerosis (MS) is the most common autoimmune disease involving the nervous system, affecting approximately 1 in 250,000 individuals in the United States. Clinically, MS is characterized by scattered central nervous system (CNS) demyelination leading to weakness, paresthesias, sensory loss and generally reduced control over neuromuscular function.

Interferon-β (IFN-β)

IFN-β is an antiviral and antineoplastic cytokine produced by fibroblasts in response to viral stimulation. It acts as an immune system modulator and is the only treatment that has substantially altered the natural history of MS in a properly controlled clinical trial (The IFN-β Multiple Sclerosis Study Group, 1993, Neurology **43**:655-61). However, it was clear from this study that, while the high dose of IFN-β (8 million international units (MIU) injected every second day) was well tolerated and had a beneficial effect on the course of relapsing-remitting MS, it was only partially effective. These patients continued to have exacerbations, although at a reduced rate and of relatively mild clinical severity.

However, this dose could not be increased, as an early pilot trial showed that increased doses (16 MIU), administered three times a week, produced unacceptable toxicity. Following administration of the two doses (1.6 and 8 MIU) used in the control clinical trial cited above, the incidence of notable side effects was low.

The most common laboratory abnormality associated with IFN-β treatment was lymphopenia unassociated with significant changes in the total white blood cell count. Other side effects noted were influenza-like symptoms and injection site reactions. *In vitro* studies have shown that IFN-β can effectively inhibit the proliferation of mitogen-stimulated peripheral blood mononuclear cells derived from both MS patients and healthy individuals. This anti-proliferative effect was associated with reduced IL-2R expression (Rudick *et al.*, 1993, Neurology, **43**:2080-7).

Extensive studies have been carried out in laboratory rats and mice to investigate the effect of IFN-β as a treatment of experimental autoimmune encephalomyelitis (EAE). EAE is the best available animal model of MS, and is a $CD4^+$ T cell-mediated inflammatory demyelinating disease of the CNS in rodents (Pettinelli & McFarlin, 1981, J. Immunol. **127**:1420-3). EAE can be induced by inoculation of susceptible animals with CNS antigens (myelin basic protein, myelin proteolipid protein, myelin oligodendrocyte glycoprotein) and adjuvants. The development of signs of EAE is associated with the infiltration of the nervous system by T lymphocytes and macrophages (Raine, 1984, Lab. Invest. **50**:608-35).

During spontaneous recovery from EAE there is a decline in the number of inflammatory cells in the nervous system (McCombe *et al.*, 1992, J. Neurol. Sci. **113**:177-86), due in part to apoptosis of T lymphocytes and macrophages in the central nervous system (Tabi *et al.*, 1994, Eur. J. Immunol. **24**:2609-17; McCombe *et al.*, 1996, J. Neurol. Sci., **139**:1-6). Recovery from EAE is also associated with production of downregulatory cytokines such as IL-10 and TGF-β (Kennedy *et al.*, 1992, J. Immunol. **149**:2496-505).

In studies by Ruuls *et al.*, 1996, J. Immunol. **157**:5721-31), EAE was induced in Lewis rats by inoculation with a synthetic MBP peptide, residues 63-88. Treatment of these rats with recombinant rat IFN-β (3 x 10^5 U/day s.c.) from day 8 to day 17 after inoculation fully protected the animals from disease during the treatment period. However, with cessation of treatment, most animals developed a protracted and remitting disease course with strongly enhanced severity. If treatment was continued to day 26, the animals did not relapse after treatment was discontinued. From these results, it would appear that discontinuation of treatment during the recovery phase of EAE is associated with rapid onset of severe paralytic disease.

One of the characteristic features of EAE is progressive weight loss during the clinical phase of the disease, which is rapidly reversed when the animals recover (Ruuls *et al.*, 1996, *supra*). Treatment with IFN-β from day 8 to day 26 inhibited weight reduction, when

compared with the control group. However, subsequent to treatment, all animals showed retardation of growth, which became evident approximately 25 days after inoculation. Failure to gain weight has also been observed as one of the most common and dose-limiting side effects of patients during IFN-β treatment for various diseases.

Using a mouse model of EAE, Yu *et al.*, 1996, J. Neuroimmunol. **64**:91-100 determined the effect of treatment with mouse IFN-β on the course of EAE in (SWR x SJL)F_1 mice, following immunization with the myelin proteolipid protein (PLP) immunodominant peptide p139-151. They showed a marked decrease in mean neurological deficit, a significant delay in exacerbation onset and exacerbation rate and a decrease in DTH. IFN-β treatment produced a long-term improvement in mean clinical score, a clear histopathological improvement and a delay in progression to disability. *In vitro,* IFN-β inhibited the proliferation of determinant-primed lymph node cells in a dose dependent manner. In conclusion, these authors noted that there was a clear histopathological improvement in the IFN-β-treated mice but that the improvement was far from a cure. A similar significant but modest long-term therapeutic effect on severity and incidence of CNS inflammation has been described in MS patients (IFN-β Multiple Sclerosis Study Group, 1993).

Early pregnancy factor (EPF).

EPF was first described in Morton *et al.,* 1974, Nature,

249:459-60 and Morton *et al.*, 1976, Proc. R. Soc. Lond., **193**:413-9.

EPF appears in maternal serum within 6–24 hr of fertilization, is present for at least the first half of pregnancy in all species studied and is essential for continued embryonic growth and survival (Morton *et al.*, 1987, Current Topics in Developmental Biology **23**:73-92; Athanasas-Platsis *et al.*, 1989, J. Reprod. Fert. **87**:495-502; Athanasas-Platsis *et al.*, 1991, J. Reprod. Fert. **92**:443-51). EPF is also an autocrine survival factor for tumour cells (Quinn *et al.*, 1990, Clin. Exp. Immunol. **80**:100-8; Quinn & Morton, 1992, Cancer Immunol. Immunother. **34**:265-71) and for regenerating liver cells following partial hepatectomy (Quinn *et al.*, 1994, Hepatology **20**:1294-302). Like many other growth factors, EPF is present in platelets suggesting that it may have a physiological role in wound healing (Cavanagh & Morton, 1994, 1994, Eur. J. Biochem. **222**:551-60).

EPF also has immunomodulatory properties. This was first proposed when it was shown that EPF is able to augment the rosette-inhibiting properties of an immunosuppressive anti-lymphocyte serum (Morton *et al.*, 1974, *supra*; Morton *et al.*, 1976, *supra*), and of anti-CD4 but not anti-CD8 antibodies (Morton *et al.*, 1982, Pregnancy Proteins, Eds. B. Grudzinskas, B. Teisner, M. Seppala, Academic Press, Sydney, 391-405). Further studies showed that EPF can suppress the delayed-type hypersensitivity (DTH) reaction to trinitrochlorobenzene (TNCB) in mice (Noonan *et al.*, 1979, Nature **278**:649-51), suppress mitogen-

induced lymphocyte proliferation (Athanasas-Platsis, 1993, PhD Thesis, The University of Queensland) and suppress IFN-γ production by EPF-binding T cells. The immunosuppressive action of EPF is mediated through the sequential induction of suppressor factors and/or lymphokines. EPF binds to CD4+ T cells, sequentially releasing two genetically-restricted suppressor factors EPF-S_1 and EPF-S_2 (Rolfe *et al*, 1988, Clin. Exp. Immunol. **73**:219-25; Rolfe *et al,* 1989, Immunol. Cell Biol. **67**:205-8). While EPF is neither species- nor strain-restricted in its action, EPF-S_1 activity is restricted to the I region of the mouse MHC and HLA-DR in human while EPF-S_2 action is localized to the Igh region.

Chaperonin 10

Amino acid sequencing of EPF purified from human platelets (Cavanagh & Morton, 1994, *supra*) has shown it to share amino acid sequence with chaperonin 10 (cpn10), a member of the heat shock family of proteins (Hartman *et al.*, 1992, Proc. Natl. Acad. Sci. U.S.A. **89**:3394-8). Thus EPF and cpn10 appear to be homologs. Cpn10 is present in a variety of organisms from bacteria to humans. The structure of cpn10 is highly conserved across mammalian species, although less so between bacteria and mammals. Cpn10 is present in mitochondria where it has a role in protein folding (Ellis & van der Vies, 1991, Annu. Rev. Biochem. **60**:321-47), and like other heat shock proteins, cellular cpn10 is upregulated during cellular stress.

Like EPF, cpn10 is active in rosette-inhibition assays and displays immunosuppressive activity such as evidenced by prolonging the

viability of allogeneic skin grafts in rats. With regard to EAE, reference is made to International Publication No. WO95/15338, wherein cpn10 was shown to delay the onset of EAE and modify the clinical features of the disease in a rat model.

Thus, it is apparent from the literature that both cpn10 and IFN-β are immunosuppressive, which property appears to be central to their usefulness in treating autoimmune diseases of the CNS, such as MS and EAE.

OBJECT OF THE INVENTION

In subsequent experiments, the present inventors have compared cpn10 and INF-β with respect to treating EAE in rats and mice, and have surprisingly discovered that cpn10 and INF-β act via different mechanisms. As a result of this discovery, it has now been realized that cpn10 and INF-β can co-operate with each other to provide an improved treatment of EAE. The implication is therefore that cpn10 and INF-β may act co-operatively to provide an improved treatment of MS in humans.

It is therefore an object of the invention to provide an improved method of treating Multiple Sclerosis.

SUMMARY OF THE INVENTION

In a first aspect, the present invention resides in a method of treating MS, including the step of administering to an individual suffering from MS a pharmacologically effective amount of cpn10 and INF-β.

In a second aspect, there is provided a pharmaceutical composition for the treatment of MS comprising:-

(i) a pharmaceutically effective amount of cpn10;

(ii) a pharmaceutically effective amount of IFN-β; and

(iii) a pharmaceutically-acceptable non-toxic carrier.

It will be appreciated that the method and pharmaceutical composition of the invention have arisen from the discovery by the present inventors that cpn10 and INF-β act in a co-operative fashion to relieve mice and rats of EAE symptoms. Accordingly, this discovery has implications for the treatment of MS in humans, EAE being a rodent experimental model of human MS. In this regard, administration of INF-β is a well-known treatment of MS, but produces side effects, particularly at higher doses of INF-β. Thus, the present invention provides a combination therapy wherein cpn10 and INF-β provide greater relief from disease symptoms than does INF-β alone, and thereby reduces the need for INF-β to be administered at doses which produce side-effects. It should also be appreciated that INF-β has become such an indispensable treatment for MS that it is highly desirable to provide co-operative treatments, such as provided by cpn10, so that patients need not be removed from INF-β treatment.

Preferably, the pharmaceutically effective amount of cpn10 is 5-60 mg.

Advantageously the pharmaceutically effective amount of cpn10 is 10-30 mg.

Preferably, the pharmaceutically effective amount of INF-β is

1-10 million International Units (MIU).

Advantageously, the pharmaceutically effective amount of INF-β is 4-6MIU.

It will be appreciated by the skilled person that the aforementioned pharmaceutically effective amounts are calculated in terms of a typical 70 kg individual. Accordingly, doses may vary depending on the weight, age, sex, general health and fitness of the individual and any other treatments to which the individual is being subjected. Furthermore, the amount of cpn10 and IFN-β administered will be interdependent with the frequency and timing of administration.

In this regard, it is contemplated that the frequency and timing of administration of cpn10 and INF-β may vary. Accordingly, it will be apparent that there are two modes of treatment contemplated within the scope of the invention:

(i) combined IFN-β and cpn10 treatment wherein each is administered at different times and/or with different frequencies; and

(ii) treatments where IFN-β and cpn10 are administered together.

Preferably, cpn10 is administered daily, although administration on a less frequent basis (e.g. twice or thrice weekly) is also possible.

As will be discussed hereinafter, INF-β is usually administered once weekly or thrice weekly, depending on the source of

IFN-β. Such administration frequencies could be maintained irrespective of how frequently cpn10 is administered. However, it is preferable that IFN-β and cpn10 are administered together daily. This latter mode of administration would be particularly suited where cpn10 and INF-β are combined in said pharmaceutical composition for subcutaneous or intramuscular injection.

Cpn10 is preferably provided in purified recombinant synthetic form. Alternatively, cpn10 could be produced by chemical synthesis. However it will be understood by the skilled person that the size of cpn10 may render chemical synthesis a less preferred option.

An example of production and purification of recombinant synthetic cpn10 using the pGEX system is provided hereinafter. Another useful approach may be to use eukaryotic expression systems, such as yeast or baculovirus expression systems, for production of recombinant synthetic cpn10. The potential advantages of such systems over bacterial expression is that cpn10 would be produced in a eukaryotic cell and without a modified N-terminus. This may lead to greater specific activity and improved stability. Examples of methods useful for recombinant protein expression can be found in Chapter 13 and Chapter 16 of CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (Eds. Ausubel et al.; John Wiley & Sons Inc., 1997 Edition), which are herein incorporated by reference.

There are currently three sources of IFN-β used in the clinical treatment of MS. These, together with other relevant information

are listed in TABLE 1.

Betaseron (or Betaferon), for example, is usually supplied by Schering in dehydrated form together with dextrose and human serum albumin as carrier. Also included is 0.54% NaCl to act as an aqueous carrier which rehydrates the dehydrated IFN-β/dextrose/human serum albumin prior to injection.

Suitably, the pharmaceutical composition comprises a pharmaceutically-acceptable carrier.

By "pharmaceutically-acceptable carrier" is meant a solid or liquid filler, diluent or encapsulating substance which may be safely used in systemic administration. The aforementioned 0.54% NaCl aqueous carrier which rehydrates dehydrated IFN-β/dextrose/human serum albumin is one example. Depending upon the particular route of administration, a variety of pharmaceutically acceptable carriers, well known in the art may be used. These carriers may be selected from a group including sugars, starches, cellulose and its derivatives, malt, gelatine, talc, calcium sulfate, vegetable oils, synthetic oils, polyols, alginic acid, phosphate buffered solutions, emulsifiers, isotonic saline, and pyrogen-free water.

Dosage forms include tablets, dispersions, suspensions, injections, solutions, syrups, troches, capsules, suppositories, aerosols, transdermal patches and the like. These dosage forms may also include controlled release devices or other forms of implants modified to act in this fashion. Controlled release of the therapeutic agent may be effected

by coating the same, for example, with hydrophobic polymers including acrylic resins, waxes, higher aliphatic alcohols, polylactic and polyglycolic acids and certain cellulose derivatives such as hydroxypropylmethyl cellulose. In addition, the controlled release may be effected by using other polymer matrices, liposomes and/or microspheres.

Pharmaceutical compositions of the present invention suitable for administration orally or by injection may be presented as discrete units such as capsules, sachets or tablets, as a powder or granules, or as a solution or a suspension in an aqueous liquid, a non-aqueous liquid, an oil-in-water emulsion or a water-in-oil liquid emulsion. Such compositions may be prepared by any of the methods of pharmacy but all methods include the step of bringing cpn10 and/or IFN-β into association with the carrier. In general, the compositions are prepared by uniformly and intimately admixing the cpn10 and/or IFN-β with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product into the desired presentation.

Preferably, cpn10 may be administered by injection or orally. More preferably, cpn10 is administered orally in the form of a tablet or capsule.

IFN-β is suitably administered by subcutaneous or intramuscular injection. Accordingly, IFN-β and cpn10 may be administered together by intramuscular or subcutaneous injection.

It will therefore be apparent that the pharmaceutical composition of the invention may be provided in the form of a kit. In such

a case, IFN-β and cpn10 may be provided in dehydrated form together with the carrier, and both rehydrated prior to injection. Alternatively, the kit may include cpn10 in tablet or capsule form, in which case cpn10 is administered orally while IFN-β is rehydrated in the carrier for administration by injection.

EXPERIMENTAL

MATERIALS

1. *Animals*

Female Lewis rats (JC strain), aged 8–10 weeks, were obtained from the Central Animal Breeding House, The University of Queensland. Female SJL/J mice aged 6–8 weeks old were obtained from the Animal Resource Centre, Western Australia. All animals were maintained on a continuous supply of mouse/rat pellets and water, in temperature- (20-22&C) and light (12 h light, 12 h dark) controlled rooms. Prior to surgical procedures, rats (average weight 170 g) were anaesthetized with intra-peritoneal anaesthetic mixture (0.2 ml) containing 10 ml ketamine (100 mg/ml), 6.2 ml xylazine (20 mg/ml), 0.8 ml atropine (600 µg/ml) and 10 ml saline (0.9% w/v). All investigations in animals were carried out in accordance with the Australian National Health and Medical Research Committee guidelines with ethical clearance from The University of Queensland Animal Experimentation Ethics Committee.

2. *Recombinant Cpn10 (rcpn10)*

Recombinant human cpn10 was prepared using the plasmid pGEX-2T expression system (Amersham Pharmacia Biotech, Uppsala,

Sweden) as described in International Publication No. WO95/15338, which is herein incorporated by reference. Briefly, using a batch technique, the glutathione S-transferase fusion protein was recovered from the cell lysate with glutathione-Sepharose 4B gel (Amersham Pharmacia Biotech), cpn10 cleaved by thrombin [0.05 M Tris-HCL pH 8.0/0.15 M NaCl/2.5 mM $CaCl_2$ buffer, 1000 units thrombin (Sigma T6884; Sigma-Aldrich, St. Louis, MO, USA); buffer 1] and recovered in the supernatant (sample 1). The gel was then washed with a high salt buffer (0.05 M Tris-HCl pH8.0/2 M NaCl; buffer 2) (sample 2). For inclusion in the lymphocyte proliferation assay (see below), cpn10 was purified on a Resource RPC column (Amersham Pharmacia Biotech); a blank run was used as a control preparation. Protein concentration of the supernatant (cpn10 sample 1) and the high salt wash (cpn10 sample 2) was estimated by the method of Lowry *et al.*, 1951, J. Biol. Chem. **193**:265-75), the purity of the preparations determined by SDS-PAGE using 15% Tris-Tricine gels (Schagger & von Jagow, 1987, Analytical Biochem., **166**:368-79), concentration of cpn10 determined by a double-antibody sandwich ELISA, and bio-activity in the rosette inhibition test (Cavanagh & Morton, 1996, Today's Life Sciences, **8**:24-7). The amino acid sequence of cpn10, prepared using the pGEX-2T expression system, is identical to human cpn10 with an additional G-S-M at the N terminus, and the molecule is non-acetylated.

3. *Recombinant murine IFN-β*

Recombinant murine IFN-β (Calbiochem, San Diego CA,

USA) of specific activity ≥8 x 10^6 units/mg protein, was supplied liquid in PBS containing 0.1% w/v BSA. Vehicle alone consisted of PBS with 0.1% w/v BSA (PBS/BSA; Sigma #9418).

4. *Myelin protolipid protein (PLP)*

The encephalitogenic PLP peptide residues 139-151 (Greer *et al.*, 1996, J. Immunol. **156**:371-9), was synthesized by the Department of Drug Design and Development, The University of Queensland.

RESULTS AND DISCUSSION

1. *IN VIVO* EXPERIMENTS WITH cpn10

1.1 *Induction of EAE and clinical assessment*

Myelin basic protein (MBP) was prepared from guinea pig brains by the method of Deibler *et al.*, 1972, Prep. Biochem., **2**:139-65. MBP in 0.9% NaCl w/v (saline) was emulsified in an equal volume of incomplete Freund's adjuvant containing 4 mg/ml *Mycobacterium butyricum*. Under anaesthesia on day 0, rats were inoculated in one hind foot-pad with 0.1 ml emulsion (50 µg MBP/rat). From day 10, rats were weighed daily and clinical signs monitored; the degree of weakness of the tail, hindlimb and forelimb was separately graded on a scale of 0 (no weakness) to 4 (total paralysis) as described by Pender, 1986, J. Neurol. Sci., **75**:317-28). A loading (x2) was added to forelimb weakness as any involvement of forelimbs represented very severe disability. On each day of examination, the total disability in each rat was recorded and results expressed as the mean disability score/group. The rats usually recovered from the disease by day 20, although in some cases a relapse was

observed in the following weeks after recovery.

1.2 *Treatment of EAE with Cpn10*

Rats were treated with cpn10 or appropriate control vehicle, starting on the day of inoculation until day 18, in doses ranging from 1 µg to 50 µg, administered every 24 or 48 hrs (see Table 2). Cpn10 sample 1 or buffer 1 were administered to rats without further treatment. Cpn10 sample 2 or buffer 2, following the addition of 1% normal rat serum, were dialyzed overnight in PBS, before administration. Cpn10 was administered either intraperitoneally (*ip*) or orally with a curved feeding needle (O/F, oral feeding) and weight and clinical score of each rat recorded to day 20. Rats receiving 50 µg/24 hrs were examined up to day 34.

Treatment of EAE rats with cpn10, either intraperitoneally or orally, resulted in a dose-dependent decrease in disability and weight loss between days 10 to 20 (Table 2). In the group of rats receiving 50 µg/day, there was no difference in the total decrease in disability between those rats that received cpn10 i.p. or orally (FIGS. 1 & 2). No incidence of relapse was observed in either of these groups, compared with a low level of relapse in the control group. An expected decrease in weight loss, compared with the control group, was seen (FIG. 3). Once the rats had entered the recovery phase, their gain in weight paralleled that of the control mice (FIG. 3).

At the doses of cpn10 tested, the animals were not completely protected against the clinical signs of EAE but it was not

possible to test higher doses due to a limited supply of cpn10. It was also considered that this molecule was not ideal due to the alteration in its N-terminus which interfered with binding to serum carrier proteins. However a significant decrease in clinical score and weight loss was demonstrated with the high dose. No relapses were observed in the cpn10-treated mice on ceasing treatment of day 17 and there was no long-term effect on weight gain. These results are in contrast to those observed following treatment of rats with IFN-β (Ruuls *et al.*, 1996, *supra*).

1.3 *Cpn10-treated rats showed inhibition of DTH reaction to MBP*

Rats were inoculated with MBP, then divided into 4 groups, 4 rats/group. Groups 1 and 4 received vehicle alone, Groups 2 and 3, cpn10 (50 µg/rat/24 hrs) orally and intraperitoneally respectively from day 0 to day 16. On day 15, ear thickness of both ears of rats was measured using an engineer's micrometer (Mitutoyo), then 20 µg MBP in 10 µl saline injected at the base of both ears of Groups 2 to 4. Group 1 rats received saline alone. After 24 hrs, both ears were again measured and ear swelling determined by subtraction.

Substantial ear swelling was observed in Group 4 rats receiving control vehicle, challenged with MBP (FIG. 4). Significantly less swelling developed in the rats that received treatment with cpn10 either orally (Group 2) or intraperitoneally (Group 3), prior to MBP challenge. Group 1 rats treated with control vehicle and challenged with saline showed no difference in ear thickness. Visually this difference was very

marked as there was a considerable inflamed red area around the base of the ear in Group 4 rats, which was not apparent in Groups 2 and 3. There was no significant difference in the ear swelling between groups 1-3. Similar results were obtained in duplicate experiments.

These results confirm that cpn10, like IFN-β, suppresses the Th1 immune response and show that cpn10, administered orally, is as effective as cpn10 i.p. in this respect.

1.4 *Total and differential white blood cell (WBC) counts*

On day 16 of treatment of EAE rats with vehicle alone, or cpn10 (50 µg/rat/24 hrs), ip or orally (n = 3/group), blood samples were obtained from the tail into EDTA for WBC counts and Wright-Giemsa-stained blood smears prepared for differential counts.

Results are shown in FIG. 5. There was no significant difference in the total WBC, lymphocyte or neutrophil counts between the three groups. Similar results were obtained in duplicate experiments.

These findings, together with those demonstrating that administration of 50 µg cpn10 daily to rats does not effect their weight gain during the recovery period, suggests that cpn10 does not have any very obvious adverse side effects. Once again, this is a characteristic not shared with IFN-β. In humans, IFN-β treatment can cause a marked lymphopoenia and, in mice, Soos *et al.*, 1995, J. Immunol. **155**:2747-53 found a 31.7% depression in lymphocyte counts 12 hrs after injection of IFN-β (10^5 U/injection).

2. *IN VITRO* EXPERIMENTS WITH Cpn10

2.1 *Effects of cpn10 on the proliferation of lymph node cells in response to MBP*

On day 10 after inoculation, 2 rats were anaesthetized, exsanguinated by cardiac puncture and the popliteal lymph node draining the inoculated limb in each rat removed under sterile conditions. The lymph nodes were chopped finely and suspended in RPMI1640 (ICN, Costa Mesa, CA, USA) containing 2 mM L-glutamine, 25 mM HEPES, 50 µM 2-mercaptoethanol, 1 mM Na-pyruvate, 50 µg/ml gentamycin, 1% heat-inactivated Lewis rat serum (incubation medium). Following removal of debris, the suspended lymphocytes were pooled, washed x 2 and cell concentration adjusted to 4×10^6 cells/ml. Before testing in the proliferation assay, HPLC-purified cpn10 was dialysed for 48 hr (with 1% normal rat serum) against PBS, then transferred into incubation medium on a NAP-5 column (Amersham Pharmacia Biotech) and filter sterilized by passage through a Millex-GV4 0.22 µm Filter Unit (Millipore, Bedford, MA, USA). Control medium (blank run from HPLC) was treated similarly. Concentration of cpn10 in the filtered preparations was confirmed in a double-antibody sandwich ELISA.

Dilutions of cpn10 were prepared in incubation medium from 100 µg/ml (10.0 µM) to 20 ng/ml (2.0 nM) (see FIG. 6) and 100 µl of each dilution dispensed in triplicate into 96-well flat-bottomed plates (Nunclon ™ Δ MicroWell Plates, Nunc, Roskilde, Denmark). MBP (50 µl, 80 µg/ml incubation medium) and prepared lymph node cell suspension (50 µl; 4 x

10^6 cells/ml) were added to each well. Control wells (6 wells each) contained either (a) cells with MBP but no cpn10 or (b) cells with no MBP nor cpn10. Plates were incubated in a humidified atmosphere at 37°C, 5% CO_2 for 72 hrs. During the last 18 hrs, each well was pulsed with 0.5 µCi [methyl-^{3}H] thymidine (Amersham Pharmacia Biotech) and incorporated radioactivity measured on a scintillation counter (EG&G Wallac, Turku, Finland). Radioactivity incorporated into wells containing cpn10 was compared to that in wells without cpn10. Parallel plates were prepared for assessment of cell viability. After 72 hrs incubation, the supernatant medium was removed, the cells resuspended in 0.1% w/v trypan blue in PBS (20 µl). Cell viability was assessed by trypan blue exclusion.

Cpn10 suppressed the MBP-induced proliferation of lymphocytes in a dose-dependent manner (FIG. 6). Thus, cpn10 can down-regulate MBP-reactive T cells, as does IFN-β (van der Meide *et al.*, 1998, J. Neuroimmunol. **84**:14-23).

2.2 *Rosette inhibition test*

Cpn10 and rat recombinant IFN-β (BioSource International, Camarillo, CA, USA) were tested for activity in the rosette inhibition test using the method previously described (Morton *et al.*, 1976, *supra*; Cavanagh & Morton, 1996, *supra*). Solutions were prepared to contain 1) 1.0 µg cpn10 in 0.5 ml PBS/0.01% BSA w/v (PBS/0.01%BSA) and 2) 1.0 µg rIFN-β in 0.5 ml PBS/0.01%BSA, then transferred into Hank's balanced salt solution/ 0.01% BSA (HBSS/0.01%BSA) on a NAP-5 column (final concentration cpn10 and IFN-β; 1.0 µg/ml). Samples were

diluted 10-fold in HBSS/0.01%BSA from 10^{-5} to 10^{-15} and the rosette inhibition titre (RIT) of each dilution determined. The cpn10 titre (log reciprocal sample dilution) was recorded as the highest dilution of a sample to give a positive result in the assay.

The cpn10 sample (1.0 µg/ml) gave a titre of 10^{-12} in the assay, while the IFN-β sample gave no activity at any concentration.

Cpn10 binds to Th1 cells, releasing genetically restricted suppressor factors, which are active in the rosette inhibition test. These results show that the mechanism whereby IFN-β suppresses the Th1 immune response differs from that of cpn10.

In the rat model, the results have shown that treatment with cpn10:

(i) decreases the disability and weight loss of animals with EAE;

(ii) suppresses the DTH reaction *in vivo;* and

(iii) down-regulates the activity of encephalitogenic (MBP-reactive) cells *in vitro*.

Thus, like IFN-β, cpn10 will suppress the Th1 response. However the means by which this response is down-regulated appears to differ.

Cpn10 is active in the rosette inhibition test, a measure of its ability to release specific genetically-restricted suppressor factors from CD4+ T cells. IFN-β has no activity in this assay.

Administration of cpn10 to rats does not affect the total

lymphocyte count, whereas Soos *et al.*, 1995, *supra* have shown that IFN-β treated mice develop a marked lymphopoenia. This has also been observed in patients receiving IFN-β treatment. Unlike treatment with IFN-β, treatment with cpn10 does not have any long term effect on the well-being of the rats as assessed by weight gain subsequent to treatment.

These results have shown that the mode of action of IFN-β and cpn10 differ but both can suppress the Th1 immune response and, in doing so, modify the symptoms of EAE. A mouse model of EAE was developed to determine if combined complementary therapy between IFN-β and cpn10 in the treatment of EAE could be achieved.

3 COMBINED INF-β AND CPN10 THERAPY

3.1 *Studies of the effect of combined cpn10 and IFN-β therapy on EAE in mice*

Female SJL/J mice (Animal Resource Centre, WA), aged 6-8 weeks old (maintained as described above) were treated with either cpn10 (sample 1) in Tris/thrombin buffer (control buffer 1) or cpn10 (sample 2) in Tris/high salt buffer (control buffer 2). The appropriate buffer was used as a control (buffer1/buffer2). Sample 2 and control buffer 2, following addition of 1% v/v normal Lewis rat serum, were dialyzed overnight in saline before administration. Induction of EAE was carried out as described by Greer *et al.*, 1996, *supra*). Mice were injected s.c. in the flank with 100 µg of PLP peptide and 100 µg of *Mycobacterium*

tuberculosis H37Ra (Difco Laboratories, USA) in an emulsion of water and CFA (Sigma, USA). Each mouse was also injected *iv* on days 0 and 3 with Bordetella pertussis bacilli (Sapphire Bioscience, Australia), 0.3 ml (1 µg/ml)/mouse. Mice were assessed clinically by daily weighing and observation and clinical disability scored as follows; 0 = no disease, 1 = decreased tail tone and slightly clumsy gait, 2 = tail atony and/or moderately clumsy gait and/or poor righting ability, 3 = limb weakness, 4 = limb paralysis, 5 = moribund state.

3.2 *Treatment of PLP-induced EAE in SJL mice with cpn10*

Preliminary experiments established that treatment of mice every 48 hrs was preferable to treatment every 24 hrs as, unlike the rats, these mice do not respond well to frequent handling. Groups (n = 6) of mice were treated with cpn10, 10 µg/mouse/48 hrs or 2.5 µg/mouse/48 hrs, or vehicle alone (buffer 1/ buffer 2) administered *ip*, commencing day 0 and continuing until day 20. Clinical score and weight were recorded daily. Total % decrease in clinical score was calculated from the area under the curve.

The results of this experiment are shown in FIG. 7. There was a significant reduction in disability in the groups receiving the cpn10 preparations, when compared with mice receiving vehicle alone. From d 10 to d 22, the % decrease in mean disability, when compared with control, for the group of mice receiving 2.5 µg/mouse/48 hrs was 48% and for those receiving 10 µg/mouse/48 hrs was 84%. Cpn10 can effectively suppress the symptoms of PLP-induced EAE in SJL mice.

3.3 *Combined treatment of PLP-induced EAE in SJL mice with cpn10 and IFN-β*

Groups of SJL mice were tested to determine if combined treatment with cpn10 and IFN-β would give greater decrease in disability than these reagents administered alone. Suboptimal doses of cpn10 (see above) and IFN-β (Yu *et al,* 1996, J. Neuroimmunol. **64**:91-100) were administered so that an additive effect could be observed. As a result of findings in preliminary experiments, IFN-β treatment was commenced on day (d) 10, following the method described by Yu *et al* (1996), and all mice received treatment with antihistamine [Pyrilamine, Sigma, USA; 10 µg/g body weight *ip* (Jose *et al,* 1994, J Exp Med, **79**:881-7)] on d 18 and d 20.

Four groups (n = 10 per group) of mice were administered preparations as described below per mouse per 48 hr period from d 0 to d 20.

Group 1: Control vehicle, 50 µl buffer 1/buffer 2 *ip* + 50 µl PBS/ BSA *sc.*

Group 2: Control vehicle, 50 µl buffer 1/buffer 2 *ip* + IFN-β *sc*, 0.5 x 10^4 U in 50 µl PBS/BSA.

Group 3: Cpn10 *ip*, 2.5 µg in 50 µl buffer 1/buffer 2 + 50 µl PBS/BSA *sc.*

Group 4: Cpn10 *ip*, 2.5 µg in 50 µl buffer 1/buffer 2 + IFN-β *sc*, 0.5 x 10^4 U in 50 µl PBS/BSA

From d 8 to d 50, mice were assessed clinically by weighing and observation as described above.

A greater decrease in disability was observed in mice receiving the combined therapy of cpn10 and IFN-β compared with those receiving cpn10 or IFN-β alone (FIG. 8, Table 3). Treatment with cpn10, IFN-β or cpn10 + IFN-β all showed a greater decrease in disability compared with that observed in mice treated with vehicle alone (FIG. 8, Table 3). Decrease in disability with the combined therapy was more marked in mice during relapse after termination of the treatment. Similar results were observed in a duplicate experiment. The effect on the disease in all three treatment groups was maintained throughout the observation period (50 days).

Thus, it is concluded that the actions of cpn10 and IFN-β are complementary in the treatment of PLP-induced EAE in SJL mice.

In humans, IFN-β is an important treatment of MS. However, the dose of IFN-β which can be safely administered is limited due to toxic side effects which accompany higher doses of IFN-β. In cases where the dose of IFN-β is limited by toxic side effects, it is proposed that cpn10 could be very effective in a combined complementary therapy, together with IFN-β, for the treatment of MS.

TABLES

TABLE 1

	Betaseron	Avonex	Rebif
Manufacturer	Schering	Biogen	Ares-Serono
Site of injection	*sc*	*im*	*sc*
Frequency of injection	thrice weekly	one per week	thrice weekly

TABLE 2

Site of cpn10 administration	Dose (µg)	Time interval of administration (hr)	Time period of examination (days)	% decrease in disability (versus vehicle alone)	*P* value
ip	1	48	10-20	nil	NS
ip	1	24	10-20	nil	NS
ip	20	48	10-20	8.2	0.252
ip	20	24	10-20	27.9	0.008
oral	20	24	10-20	14.7	0.02
ip	50	24	10-20	36.1	0.001
oral	50	24	10-20	32.2	0.001
ip	50	24	10-35	49.5	0.001
oral	50	24	10-35	46.1	0.001

TABLE 3

	IFN-β	cpn10	cpn10 + IFN-β
primary disease day 9 to day 21	28.4	42.3	46.2
first relapse day 22 to day 31	48.0	67.2	74.6
second relapse day 32 to day 40	48.8	64.7	71.8

LEGENDS

TABLE 1

List of IFN-β preparations currently used in the treatment of MS: *sc* = subcutaneous injection; *im* = intramuscular injection.

TABLE 2

Decrease in disability in EAE rats (as % of control) following treatment with cpn10. Rats were inoculated with MBP in one rear footpad on day 0. Cpn10 (n=7/group) or vehicle alone (buffer 1/buffer 2; n=6/group) was administered intraperitoneally (ip) or orally at the time intervals indicated commencing day 0 until day 17. Clinical signs were monitored daily (see methods) from day 10 to day 20 or to day 35 and the area under the curve calculated. The results are expressed as decrease in the total clinical score in the test group as a % of the total disability score in the group receiving vehicle alone, tested concurrently. Data were analyzed using Student's *t* test.

TABLE 3

Decrease in disability in EAE mice following treatment with cpn10, IFN-β or a combination of both. All values refer to % decrease in disability as a % of control group. All values are significantly different at the $p<0.001$ level, from that of the control group and other test groups except: (i) primary disease cpn10 v cpn10 + IFN-β where p=0.15; (ii) primary disease cpn10 v IFN-β where p=0.005; (iii) second relapse cpn10 v IFN-β where p=0.03; (iv) second relapse cpn10 v cpn10 + IFN-β where p=0.07;

and (v) second relapse IFN-β v cpn10 + IFN-β where p=0.002.

FIG. 1

Recombinant cpn10 administered intraperitoneally to EAE rats commencing day of innoculation with MBP. Values represent mean disability ± standard error of the mean (SEM). Cpn10 treatment significantly decreased the total disability score ($p < 0.001$; paired Student's *t* test).

FIG. 2

Recombinant cpn10 administered orally to EAE rats commencing day of innoculation with MBP. Values represent mean disability ± standard error of the mean (SEM). Cpn10 treatment significantly decreased the total disability score ($p < 0.001$; paired Student's *t* test).

FIG. 3

Recombinant cpn10 pGEX administered intraperitoneally (i/p) or orally (O/F) to EAE rats commencing day of inoculation with MBP. Values represent % weight loss per group (compared to wieght at day 10).

FIG. 4

Delayed type hypersensitivity (DTH) reaction to MBP in rats, administered with cpn10 intraperitoneally (ip) or orally, or treated with control vehicle, commencing day of innoculation with MBP. Four groups of rats (n=4 per group) were inoculated with MBP on day 0 and received cpn10 (50 μg/rat) by oral feeding (O/F; group 2) or ip (group 3) or vehicle alone ip (groups 1 and 4) daily from day 0 to 16. On day 15, ear thickness of both ears of rats was measured, then 20 μg MBP in 10 μl saline was injected into the

base of both ears in groups 2-4. Group 1 received injections of saline. After 24 hr, both ears were again measured and ear swelling determined by subtraction: *** $p<0.001$; **$p=0.003$ compared to group 4 (Student's *t* test).

FIG. 5

Effect of cpn10 administered i.p daily or orally to rats (n=3), commencing day of innoculation with MBP (day 0), on total and differential leukocyte counts. Counts were obtained on day 16.

FIG. 6

Suppression of MBP-stimulated lymphocyte proliferation *in vitro* by cpn10. Lymphocytes were recovered from draining lymph nodes of rats 10 days after innoculation with MBP. Dilutions of cpn10 were prepared to give a final concentration on the plate and 100 µl dispensed in triplicate. To each well was added 50 µl washed lymphocytes (4×10^6/ml) and 50 µl MBP (80 µg/ml). Control wells contained either: a) cells with MBP, no cpn10, or b) cells with no MBP, no cpn10. Plates were incubated for 72 hr and pulsed during the last 18 hr with 0.5 µCi [*methyl*-^{3}H] thymidine. Proliferation was assessed by measuring incorporated radioactivity in cells incubated with or without cpn10. ***$p<0.001$; **$p=0.005$ (Student's *t* test).

FIG. 7

Decrease in mean disability score of EAE in SJL mice treated with recombinant cpn10 compared with mice treated with vehicle alone. EAE was induced in mice (n=10 per group) with PLP peptide p139-151 and

cpn10 (10 μg/mouse/48 hr or 2.5 μg/mouse/48 hr) or vehicle alone administered ip from day 0 to day 20. From day 8, weight and clinical signs were monitored daily and total disability of each mouse recorded. The results are expressed as mean disability score/group. The total disability (day 10 to day 22) in the mice receiving cpn10 was significantly reduced in both groups, when compared with the group receiving vehicle alone ($p < 0.001$, paired Student's *t* test) as was that of mice receiving 10 μg/mouse/48 hr compared with mice receiving 2.5 μg/mouse/48 hr.

FIG. 8

Cpn10 and INF-β administration to SJL/J EAE mice. EAE was induced in SJL mice (n=10 per group) with PLP peptide p139-151. Cpn10 (2.5 μg/mouse/48 hr), IFN-β (0.5×10^4 U/mouse/48 hr), cpn10 (2.5 μg/mouse/48 hr)+ IFN-β (0.5×10^4 U/mouse/48 hr) or vehicle alone was administered to mice (n=10 per group) ip (cpn10) or sc (IFN-β) from day 0 to day 20. From day 8, weight and clinical signs were monitored daily and total disability of each mouse recorded. The results are expressed as mean disability score per group. The total disability (day 10 to day 22) in the mice receiving cpn10 and/or IFN-β was significantly reduced in the three test groups, when compared with the group receiving vehicle alone ($p<0.001$ according to paired Student's *t* test; see Table 2).

DATED this Twentieth day of January 1999.

THE UNIVERSITY OF QUEENSLAND

By its Patent Attorneys

FISHER ADAMS KELLY

FIGURE 1

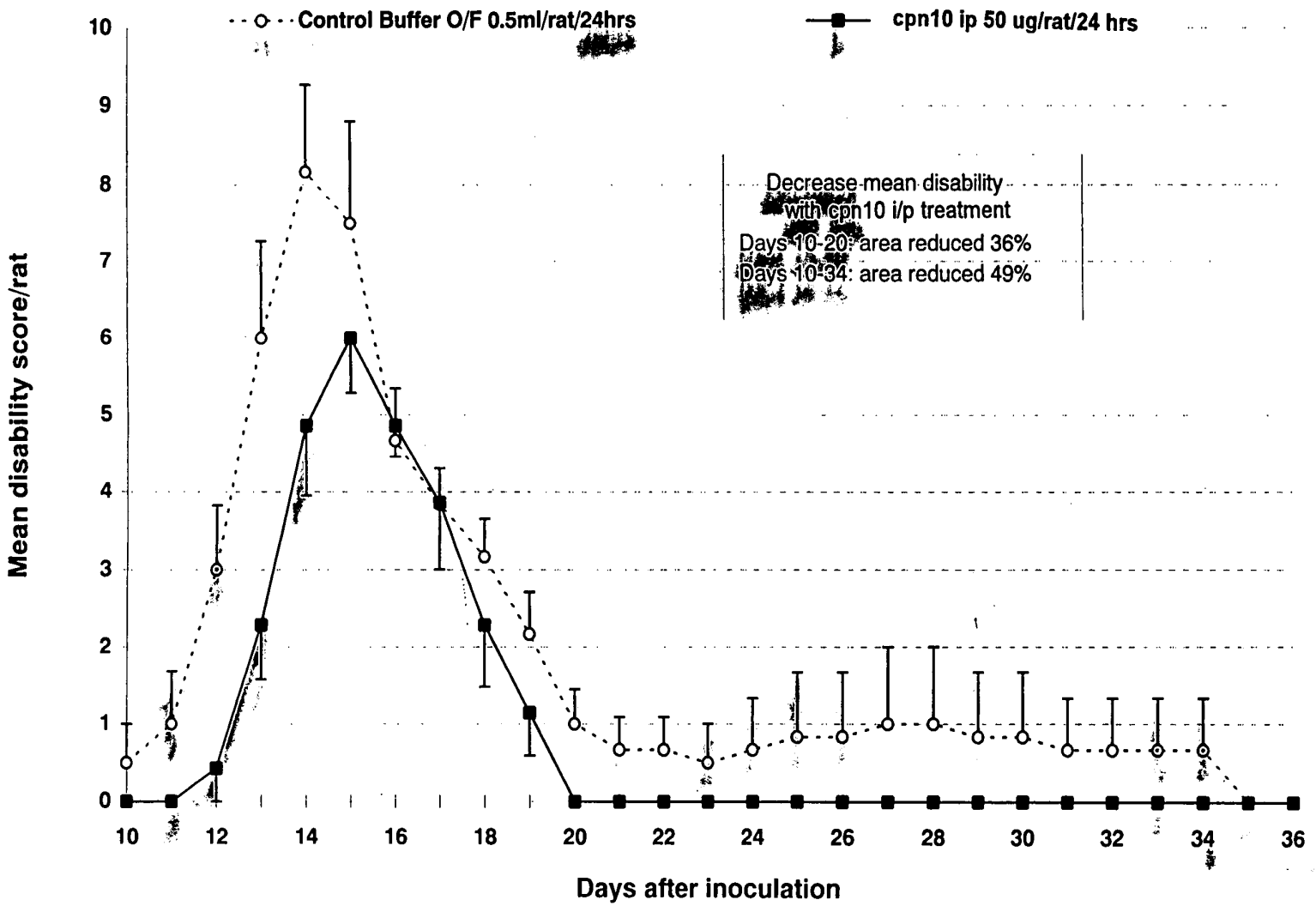

Control Buffer O/F 0.5ml/rat/24hrs
cpn10 ip 50 ug/rat/24 hrs
Decrease mean disability with cpn10 i/p treatment
Days 10-20: area reduced 36%
Days 10-34: area reduced 49%
Mean disability score/rat
Days after inoculation
0
1
2
3
4
5
6
7
8
9
10
10
12
14
16
18
20
22
24
26
28
30
32
34
36

FIGURE 2

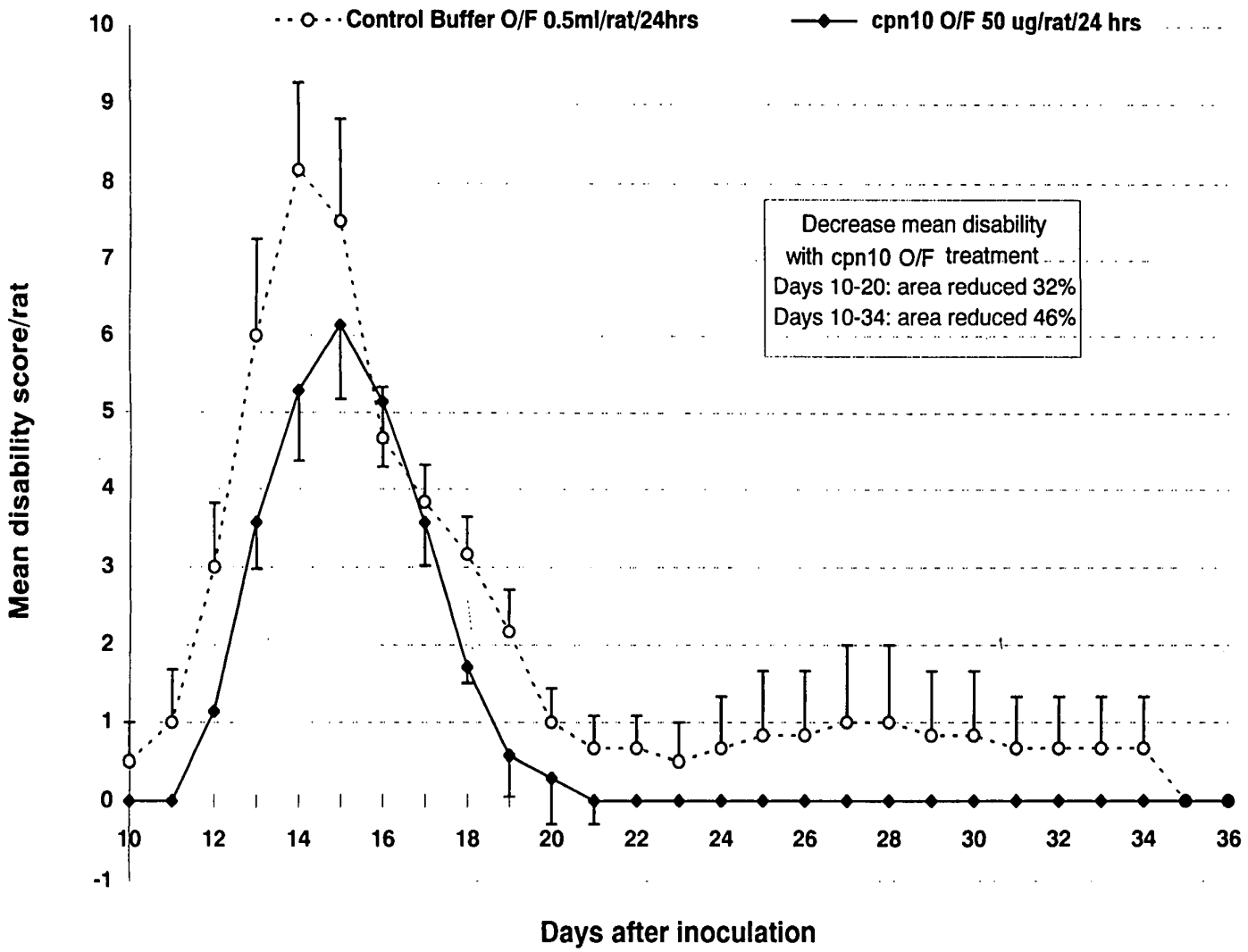

Control Buffer O/F 0.5ml/rat/24hrs
cpn10 O/F 50 ug/rat/24 hrs
Decrease mean disability
with cpn10 O/F treatment
Days 10-20: area reduced 32%
Days 10-34: area reduced 46%
Mean disability score/rat
Days after inoculation
10
9
8
7
6
5
4
3
2
1
0
-1
10
12
14
16
18
20
22
24
26
28
30
32
34
36

FIGURE 3

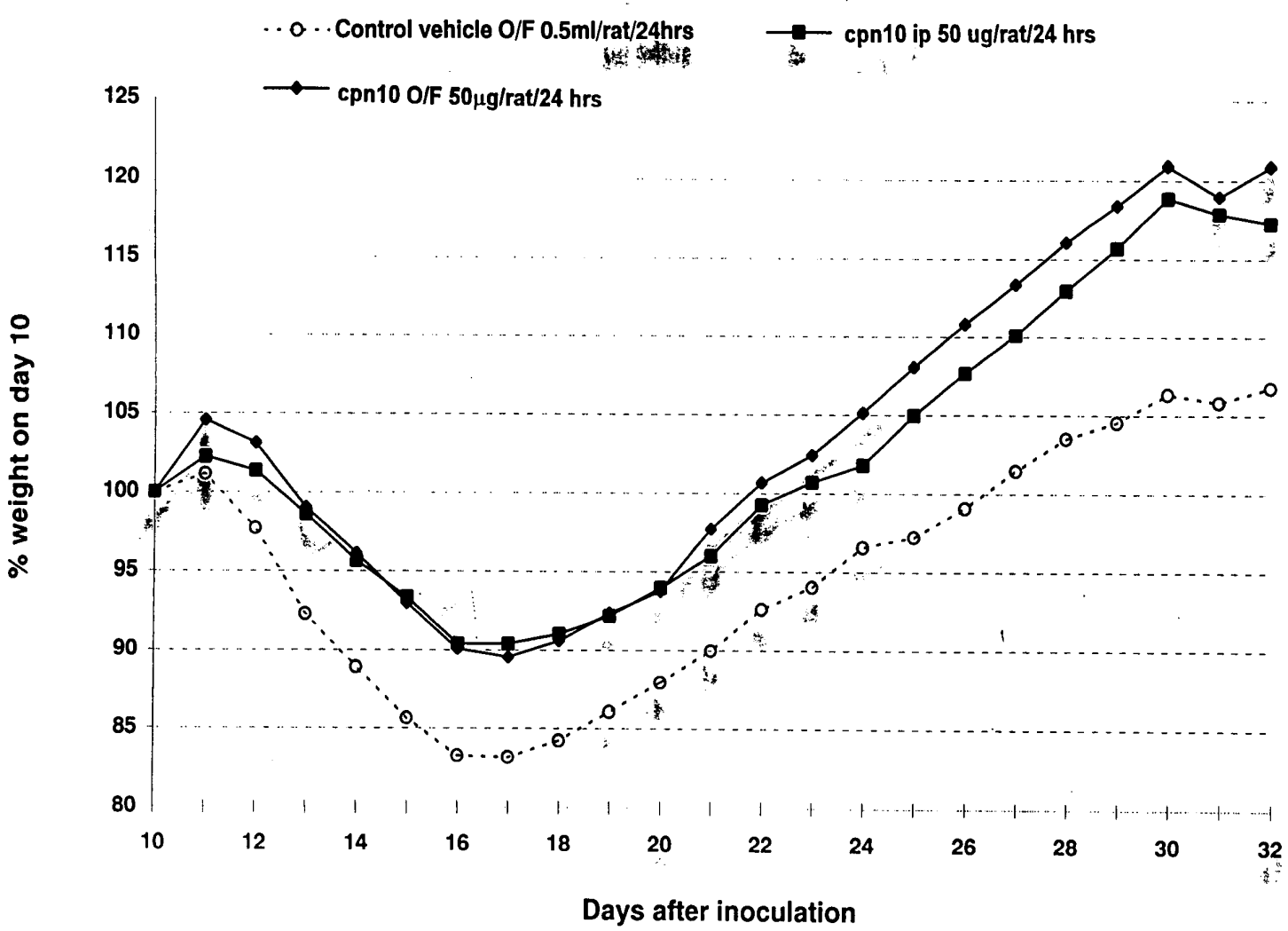

Control vehicle O/F 0.5ml/rat/24hrs
cpn10 ip 50 ug/rat/24 hrs
cpn10 O/F 50µg/rat/24 hrs
% weight on day 10
125
120
115
110
105
100
95
90
85
80
10
12
14
16
18
20
22
24
26
28
30
32
Days after inoculation

FIGURE 4

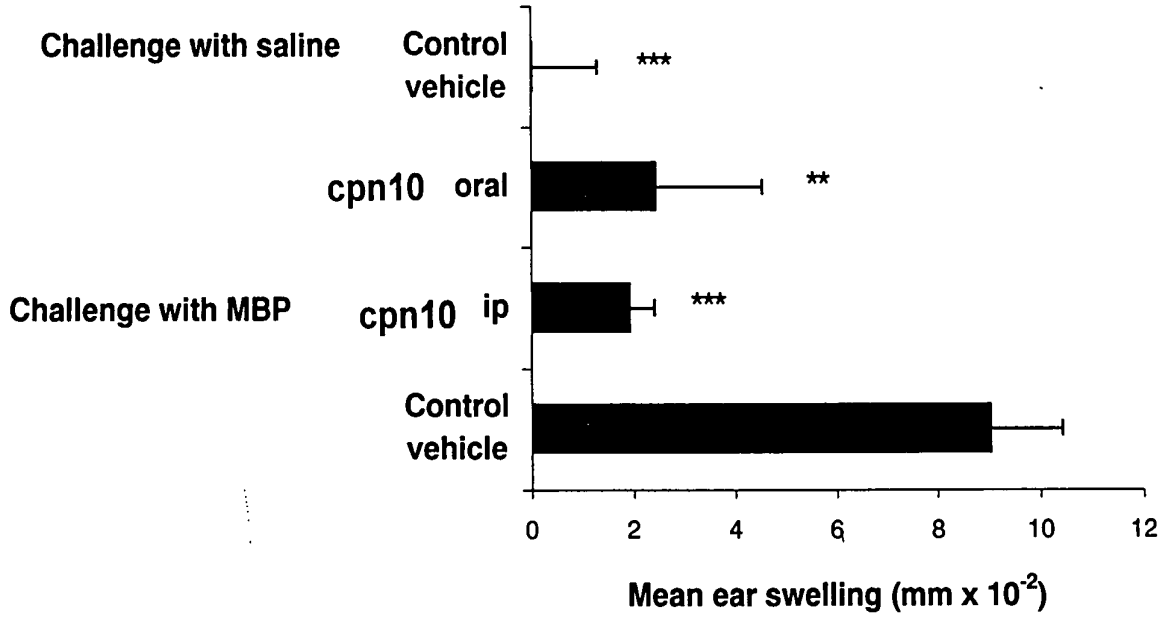

Challenge with saline
Control vehicle
***
cpn10 oral
**
Challenge with MBP
cpn10 ip
***
Control vehicle
0
2
4
6
8
10
12
Mean ear swelling (mm x 10-2)

FIGURE 5

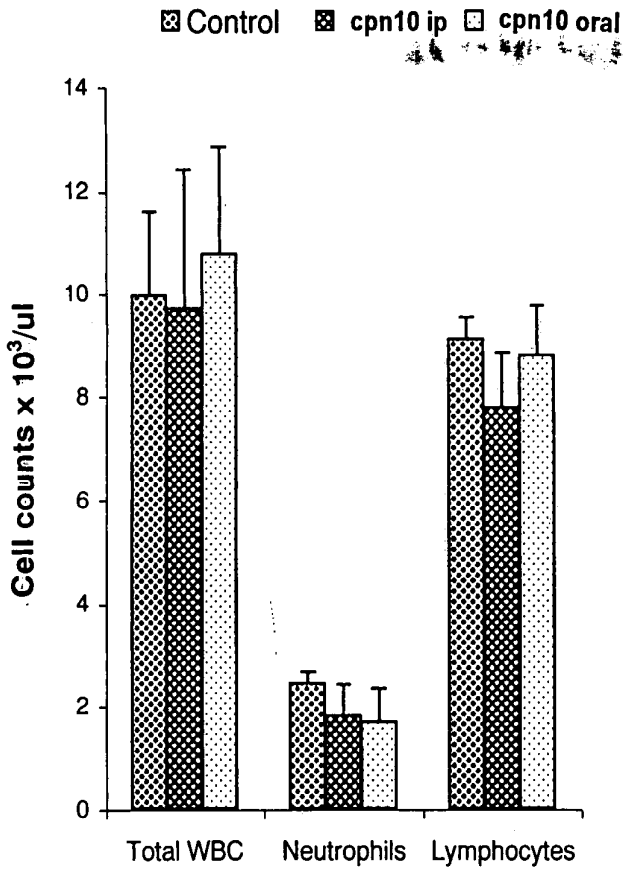

Control
cpn10 ip
cpn10 oral
Cell counts x 10³/ul
14
12
10
8
6
4
2
0
Total WBC
Neutrophils
Lymphocytes

FIGURE 6

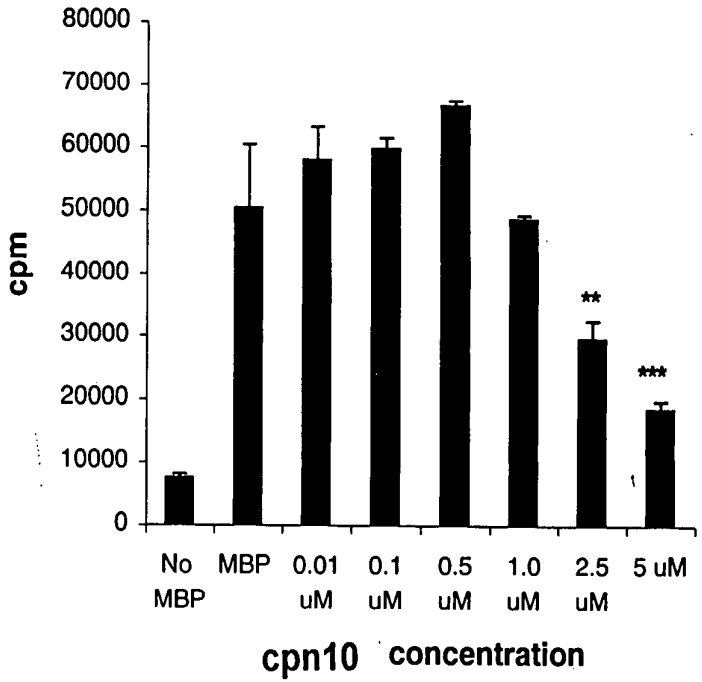

80000
70000
60000
50000
40000
30000
20000
10000
0
cpm
**
***
No MBP
MBP
0.01 uM
0.1 uM
0.5 uM
1.0 uM
2.5 uM
5 uM
cpn10 concentration

FIGURE 7

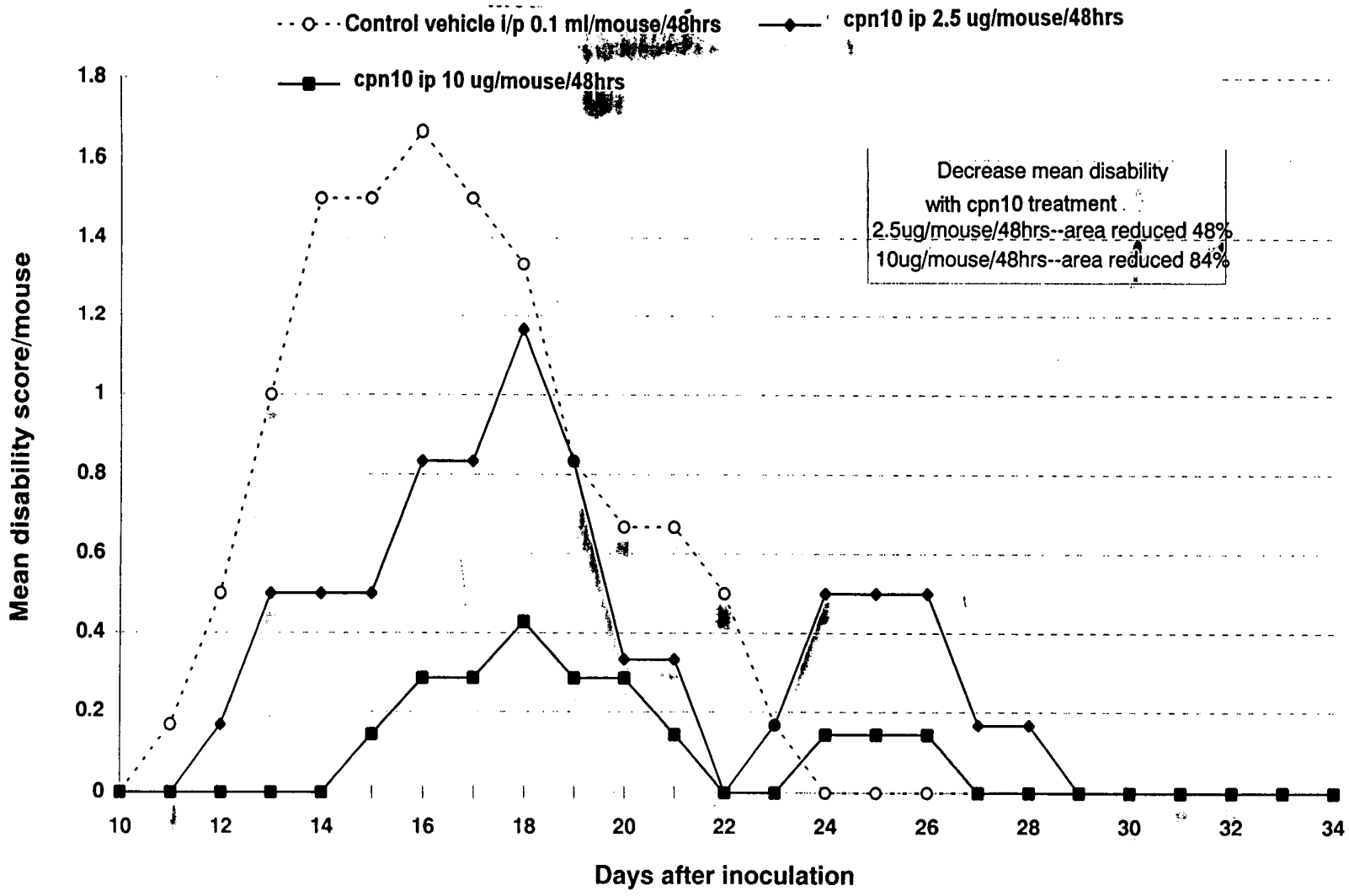
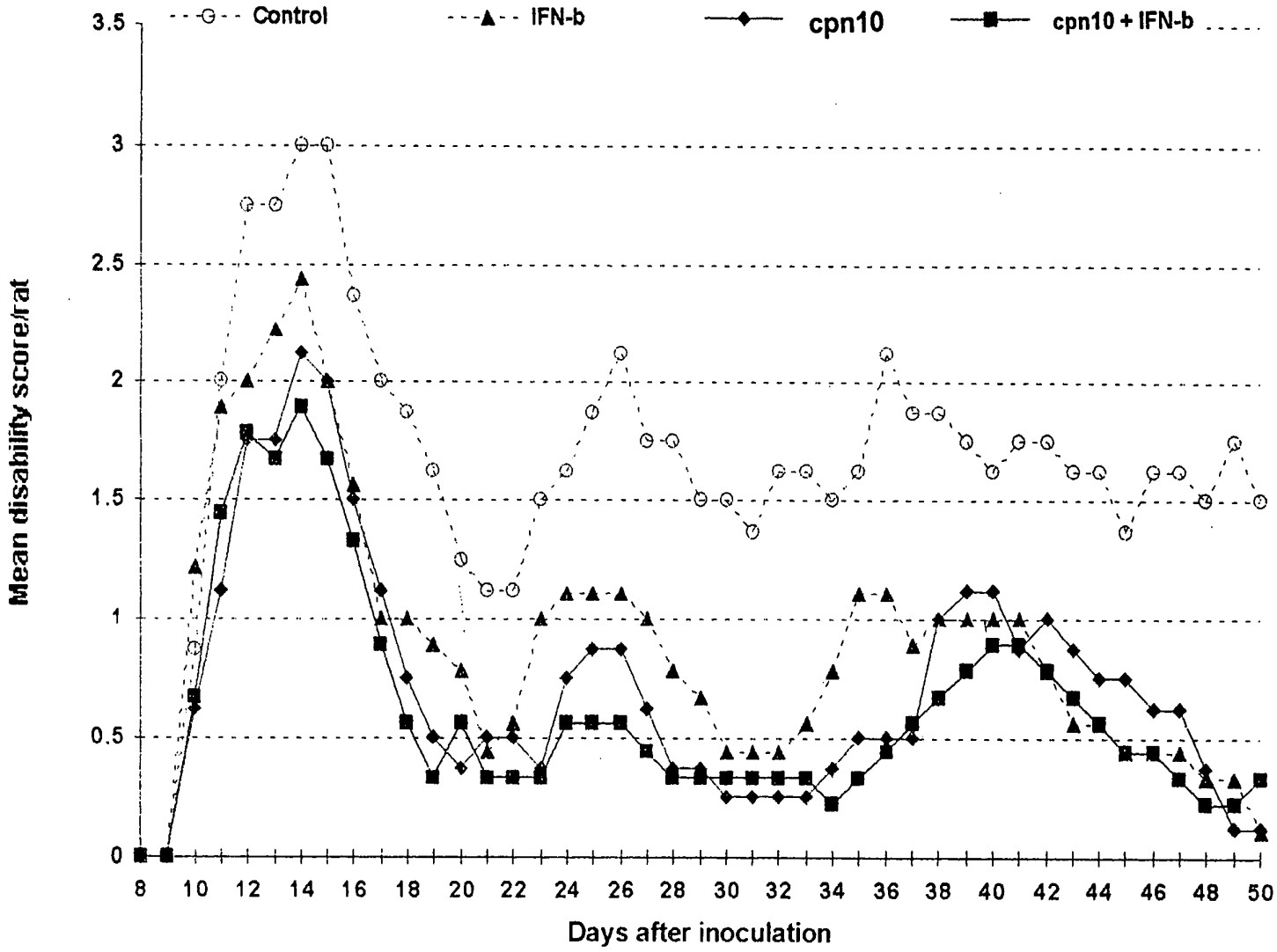

Control vehicle i/p 0.1 ml/mouse/48hrs
cpn10 ip 2.5 ug/mouse/48hrs
cpn10 ip 10 ug/mouse/48hrs
Decrease mean disability
with cpn10 treatment
2.5ug/mouse/48hrs--area reduced 48%
10ug/mouse/48hrs--area reduced 84%
Mean disability score/mouse
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
10
12
14
16
18
20
22
24
26
28
30
32
34
Days after inoculation

FIGURE 8


Control
IFN-b
cpn10
cpn10 + IFN-b
Mean disability score/rat
3.5
3
2.5
2
1.5
1
0.5
0
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
42
44
46
48
50
Days after inoculation